SALINITY AND THE COMMERCIAL PRODUCTION OF BETA-CAROTENE FROM *DUNALIELLA SALINA*

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The ability to grow at high salinity, with the concomitant production of high levels of beta-carotene and glycerol, has made the unicellular green alga *Dunaliella salina* Teodoresco (Volvocales; Chlorophyta) very suitable for commercial exploitation. This paper presents some results from three years of pilot plant trials with the *D. salina*/beta-carotene process and considers in particular the role salinity plays in various aspects of the utilization of this algal resource.

Pilot plant studies were commenced in 1980 by Roche Algal Biotechnology, a division of Roche Products (Australia) Pty. Ltd. At the end of 1983 the project was taken over by Wesfarmers, an Australian company, with the aid of Australian Government funding. The present operating schedule provides for the first *Dunaliella*-derived products to be marketed in two years' time, with other products from *Dunaliella* and other algae becoming available thereafter.

The pilot plant is situated at Hutt Lagoon, about 450 km north of Perth in the sub-tropical Murchison region of Western Australia (Fig. 1). This area has relatively high insolation and most rainfall is limited to winter; the normal annual rainfall is 478 mm. Hutt Lagoon is a seasonally dry salt lake, about 10 km x 2 km in size, separated from the sea by sand dunes. The pilot plant ponds have been built on the eastern edge of the salt lake on the lake bed and the ancilliary facilities are located on the adjacent shore (Borowitzka et al. 1984).

Hutt Lagoon supports its own seasonally abundant *Dunaliella* populations, and our pilot plant process cultivates the highly carotenogenic *D. salina* normally found in the lagoon waters. This *D. salina* appears to be identical with the *D. bardawil* of Ben-Amotz et al. (1982), since both fit the type description of *Dunaliella salina* (Teodoresco, 1905). The non-carotenogenic *Dunaliella* species referred to as *D. salina* in some publications by Ben-Amotz and Avron (Ben-Amotz and Avron, 1983) is comparable to our *D. parva* (Lerche, 1938).

Salinity is important to four areas of commercial production of *Dunaliella*. These are: 1) maintenance of the *D. salina* monoculture; 2) rate of biomass production and carotenogenesis; 3) cell behavior; and 4) processing the biomass to a marketable product.

1. MAINTENANCE OF THE *DUNALIELLA SALINA* MONOCULTURE

Sodium chloride concentration is the major selective factor acting on the populations of algae and other organisms in the open air pilot plant ponds. Other factors such as high incident irradiation, ionic composition of the brines, nutrient levels

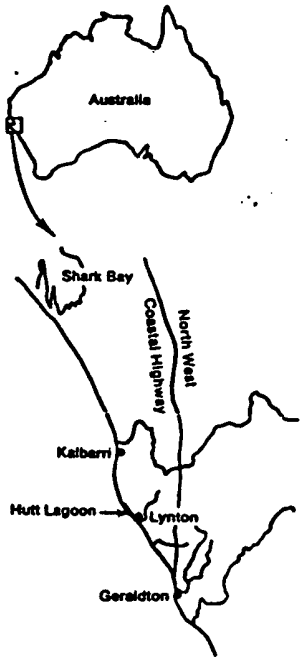

Fig. 1. Locality map of Hutt Lagoon. The pilot plant is situated on the eastern shore of Hutt Lagoon (28 ° 10'S, 114 ° 16'E).

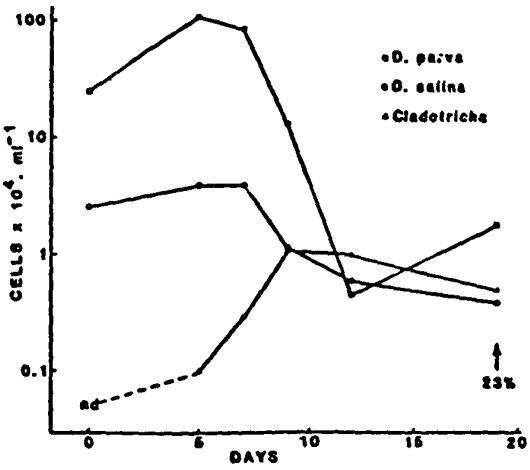

Fig. 2. Cell counts of *D. salina*, *D. parva* and the protozoan *Cladotricha sigmoidea* in a pilot plant pond at 17% salinity. Day 0 was the 24 February 1982. The salinity was allowed to increase by evaporation after day 14.

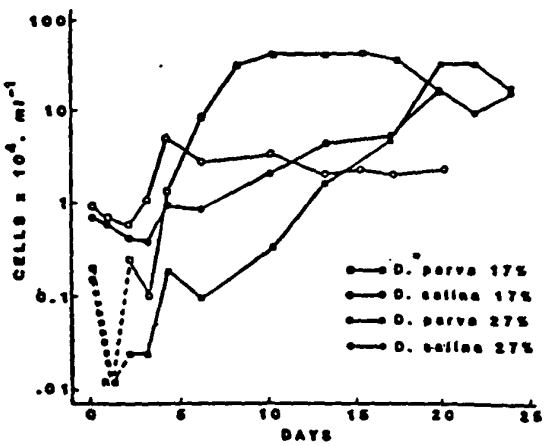

Fig. 3. Cell counts of *D. salina* and *D. parva* in pilot plant ponds maintained at 17% and 27% salinities respectively in late autumn. Day 0 was 5 May 1982.

and water temperature also play a part.

The major eukaryotic heterotrophs of the highly saline waters of Hutt Lagoon and the pilot plant ponds are protozoa. Post et al. (1983) have recorded 14 species of ciliates, 10 zooflagellates and 4 sarcodines active at salinities greater than 15% at Hutt Lagoon. A few species were still active in saturated brine. At least four species of protozoa (three ciliates and one amoeba) are important predators of *Dunaliella*, but their growth and survival is limited at high salinities where *Dunaliella* cells still actively grow and divide. For example we have observed a rapid increase in the numbers of the ciliate *Cladotricha sigmoidea* in a pond at 17% salinity. This resulted in a dramatic reduction of the cell densities of *D. parva* and *D. salina* (Fig. 2). No such predation effects have been observed in ponds maintained at greater than 20% salinity, although in the above example *Cladotricha* remained common even when the salinity was raised to 23%. Control of protozoan populations therefore requires careful management of pond salinities, especially during periods of rainfall in order to maintain high cell numbers of *D. salina*.

Dunaliella salina coexists with several non-carotenogenic *Dunaliella* species and under certain circumstances may compete with them for light and nutrients. The non-carotenogenic species, *D. parva* and *D. viridis*, generally have faster growth rates and photosynthetic rates than *D. salina* at low salinities. At higher salinities, between 25% and 32%, the relative differences between carotenogenic and non-carotenogenic *Dunaliella* species diminishes, but under the environmental conditions of late autumn we found that the non-carotenogenic species still grew faster (Fig. 3). In contrast, in summer conditions we have grown and maintained ponds with high levels of *D. salina* and low levels of *D. parva* at salinities of 25-30% (Fig. 4). We believe that the determining factor is light intensity, and that at high light intensity and high salinity *D. salina* grows faster and maintains higher cell densities than *D. parva*. Ben-Amotz and Avron (1983) have also found similar growth rates with what we believe to be the same two species under non-limiting light conditions and at a salinity greater than 21% (= 3.5 M NaCl). The carotenoid pigments prevent photoinhibition in *D. salina* at high light intensities (Borowitzka et al. 1984) typically encountered at Hutt Lagoon in summer. Red *D. salina* cells are therefore able to photosynthesize at their maximal rate at these light intensities, whereas photosynthesis in the non-carotenogenic species appears to be inhibited by the high light intensity. The beta-carotene may play a direct part in protecting the chloroplasts against photosensitized oxidations (Krinsky 1979), or just reduce the amount of light incident on the photosystems in the thylakoid membranes by acting as a light filter. The latter seems most likely since much of the carotenoid in the red cells is localized in small droplets at the periphery of the chloroplast (Borowitzka et al. 1984).

Light intensity and salinity also affect the vertical migration of the two major *Dunaliella* species.

Salinities in this paper are given as % NaCl (w/v).

At approximately 25% salinity in conjunction with high light intensity, *D. parva* cells migrate to the bottom sediments of the pond, whereas *D. salina* cells remain in the water column (Fig. 4). At saturation point (32% salinity) *D. salina* are often observed also to migrate to the bottom. This difference in behavior can be exploited to maintain a high *D. salina/D. parva* ratio.

Halophilic bacteria constitute another group of potential competitors and pathogens, however our results to date show them not to be a great problem. However, if a bloom of halophilic bacteria were initiated by a large-scale loss of glycerol from the *Dunaliella* population, the increased bacterial numbers might inhibit *Dunaliella* growth by reducing light penetration into the water column. On the other hand the bacterial population of the ponds may actually enhance algal growth by regenerating nutrients such as nitrogen (Rodgers & DePinto 1981).

2. RATE OF BIOMASS PRODUCTION AND CAROTENOGENESIS

As discussed in the previous section, it appears that the combination of both high incident irradiation and high salinity is required to give *D. salina* an advantage in photosynthetic and growth rate over competitor photosynthetic organisms.

The extent of carotenoid pigment accumulation depends on high salinity, high temperature and high incident irradiation (Aasen et al. 1969). Other factors increasing carotenoid accumulation include nutrient limitation, particularly of nitrate (Mil'ko 1963) and there is an inverse relationship between growth rate and carotenoid production in the biomass with respect to environmental factors such as salinity (Borowitzka *et al.* 1984). This inverse relationship combined with the fact that increasing the salinity from 15% to 25% increases the beta-carotene content from less than 10 to 260 mg·g cell protein^{-1} in 4 days, suggests a 2-stage batch process for a commercial plant (Borowitzka et al. 1984). In such a process the first stage would be designed to produce maximum biomass under optimal conditions of low salinity and high nutrient concentration. The second stage then features accumulation of carotenoid (with little change in biomass) under conditions of high salinity and limiting nutrients.

Initial trials of such a process led us to abandon it because of:

(a) The appearance of protozoa in the low salinity, first stage growth ponds, followed by the occasional decimation of the algal population;

(b) The occasional overgrowth by the non-carotenogenic *Dunaliella* species which are always present in the ponds, in spite of the large inocula of *D. salina* used. This occurred particularly when incident irradiation was low during the first stage of growth due to cloudy weather, as well as the fact that non-carotenogenic species accumulated in bottom sediments, from which they emerged when conditions were favorable for them.

(c) The increased labour and longer elapsed time per unit yield in the batch process compared with continuous or semi-continuous process made the batch process less commercially viable.

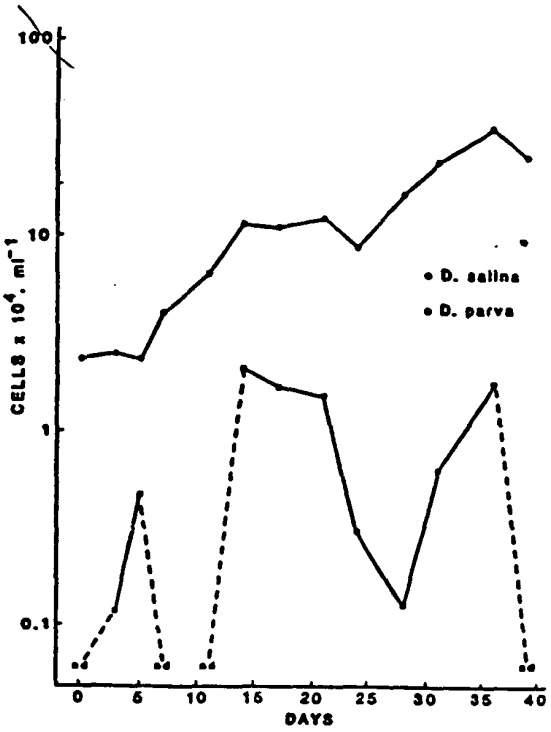

Fig. 4. Cell counts of *D. salina* and *D. parva* in a pilot plant pond maintained at 25-30% salinity. Day 0 was 2 February 1983. Cells were sampled only in the water column and the large changes in numbers of *D. parva* are largely caused by migration of the cells to and from the bottom of the pond.

Not only does the extent of carotenoid production depend on salinity in combination with other factors, but the chemical species of carotenoids also vary. For example, when *D. salina* were grown in nutrient-limited culture at 25% salinity or greater, more than 95% of the carotenoids were in the form of beta-carotene, with the remaining 5% consisting mainly of alpha-carotene. However, when *D. salina* cells were grown at a faster growth rate in 10% salinity medium enriched with bicarbonate, 71% of the

carotenoid was in the form of the oxygenated carotenoids, lutein, zeaxanthin and canthaxanthin. The total carotenoid content of the cultures grown at 10% salinity was only 3.9% of dry weight, while the total carotenoid content of the 25% salinity culture was 14% of dry weight. Varying nutrient concentration, light intensity and salinity within the extremes of these examples produced intermediate carotenoid levels with the composition of the carotenoids also varying between the above limits.

3. CELL BEHAVIOR

We believe that a commercially viable harvesting procedure for *D. salina* can usefully exploit aspects of the behavior of the cells, rather than treating the cells simply as inert particles in solution. Harvesting *Dunaliella* species from concentrated brine solutions is a greater problem than with filamentous algae, or unicellular algae in dilute solutions. *D. salina* cells are ovoids of 25 x 12-15 μm size. They are motile, flexible, and neutrally buoyant in a brine growth medium with high specific gravity. Their flexibility, due to the lack of a rigid cell wall, allows them to pass through 5 μm filters. Use of diatomaceous earth as a filter-aid traps the cells more efficiently (Ruane 1974a) but renders clean, whole cell recovery nearly impossible. However direct solvent extraction of carotenoids from the filter aid is possible (Ruane 1974b).

The neutral buoyancy of the *Dunaliella* cells in a solution with a high specific gravity means that harvesting by centrifuging requires a very high energy input. High-pressure filtration, with and without filter aids, or high speed centrifugation damages the cells, and leads to carotenoid loss through oxidation.

Several harvesting procedures, which exploit high salinity dependent physiological and behavioral characteristics of *D. salina* have been proposed. One sophisticated proposal for use of salinity-dependent buoyant properties employs stationary or moving salt gradients in the harvest of *Dunaliella* (Bloch et al. 1982). This process uses the fact that the algal cells tend to be trapped and concentrated at the interface between brine layers of different salinity, presumably in the narrow band of brine that matches the specific gravity of the cells. This *Dunaliella* cell concentration effect is frequently observed in natural salt lakes and ponds such as Hutt Lagoon, when rainwater causes stratification of the brines. This phenomenon has also been reported by Massyuk (1961).

D. salina cells are also positively phototactic. Salinity affects phototaxis in at least two ways. Firstly, flagella movement decreases with increasing salinity, probably as a result of the increased metabolic load of osmoregulation and ionic exclusion. Movement ceases entirely for a period of at least one hour following a salinity increase or decrease (Brown & Borowitzka 1979). Secondly, under some circumstances salinity-dependent carotenoid accumulation may also reduce the phototaxis because of the carotenoid droplets in the chloroplast reducing the amount of light reaching the eyespot.

Construction of suitable harvesting devices could allow exploitation of phototaxis as an inexpensive and specific concentration step, preliminary to harvest. Several such devices are mentioned in the patent of Kessler (1982). They employ floating rafts holding vertical fibres to trap *Dunaliella* cells into vertical movement paths. This patent also states that the devices also cause concentration of *Dunaliella* at the liquid surface in the dark, exploiting some phenomenon as yet unknown, but proposed to be related to the effect of the number and type of cell collisions on net movement.

Australia's CSIRO, jointly with Cockajemmy Ltd., have patented a method of exploiting the salinity dependent hydrophobicity of the *D. salina* cell membrane (Curtin & Snook 1983) as a harvesting procedure. Essentially this method states that *Dunaliella* species may be harvested from a brine containing 3 M NaCl or more (about 18%) by contact with hydrophobic substances to which the cells adsorb. By washing with fresh water or brine with a lower salinity, the adsorbed algal cells are released. The physiological basis for the stated salinity-dependent membrane properties is not known. The system is currently under pilot study by its inventors in Australia.

For development of further procedures, it should be noted that low salinity dependent characteristics may also be exploited during harvesting without compromising final carotenoid yields. Providing salinity is reduced in a manner avoiding damage to the cells, the metabolic breakdown of carotenoids in response to salinity reduction follows a much slower course than the reverse process. The metabolic loss of carotenoid following transfer of the algal cells from high salinity to low salinity was found to be negligible over a period of 4 days. A 50% loss of carotenoid occurred only after 40 days after transfer from the high salinity to the lower one (Borowitzka et al. 1984).

4. PROCESSING THE BIOMASS TO A MARKETABLE PRODUCT

Marketable products from *D. salina* biomass include carotenoid- and protein-rich algal meals for animal feeds, pure carotenoids for food and feed

applications, and glycerol for use in the food, cosmetic and explosive industries.

The salt content of harvested algal slurry must be adjusted if the product is to be a dried algal meal. Some addition of salt to prepared animal feeds can be desirable, but the acceptable level varies with each feed formulation and the type of animal to be fed. Washing the slurry or meal to remove excess salt has the further benefit of removing glycerol. The presence of excess glycerol may render the meal sticky and hygroscopic and thus difficult to store and process; after removal, recovery of glycerol from the wash may be feasible thus extending the product range.

Extraction and purification of carotenoid pigments using, for example, hexane, are not affected by the presence of salt in the initial algal slurry. The concentration of salt is therefore of no importance if a pure carotenoid product is desired.

Finally, the high salinity of the brines in which the algae are grown and from which they are harvested, also means a much greater level of equipment corrosion and even abrasion due to the presence of salt crystals. This increases the overall cost of the *Dunaliella* process due to higher plant maintenance and replacement costs.

APPLICATION OF THE PRINCIPLES TO A COMMERCIAL PROCESS

The practical expression of the principles outlined above has been under test at the Hutt Lagoon pilot plant since 1980.

In order to maintain the *D. salina* monoculture, and an optimum balance of biomass and carotenoid production, salinity is manipulated within the range 25%-32% in the growth ponds, in response to the observed proportions of carotenogenic to non-carotenogenic species. To obtain the greatest population stability "stock" (inoculum) ponds are maintained at near saturation point, 32% salinity.

High salinity brines (usually saturated) are obtained form the surface waters of Hutt Lagoon when the lake contains water, or from wells in the lake bed when it is dry. The chemical composition of these brines reflects their marine origin and subsequent deposition and resolution of salts. Initial pond salinity is set by diluting these brines with freshwater or, potentially, seawater.

Maintenance of the desired salinity in the open ponds then requires: (a) continual replacement of evaporation losses; (b) removal of rainwater before wind mixes the freshwater layer at the pond surface into the pond, diluting the brine, and (c) addition of solid salt to compensate for dilution following wind mixing of rainwater. Option (c) is generally only required during mid-winter rains or following a cyclone. In practice, high evaporation rates following infrequent summer rains restore the desired salinity within a short period.

The establishment and maintenance of open monocultures is the essential first step in micro-algal biotechnology. Once the monoculture system consisting of the selective environment and its organism, is established, such factors as biomass and product yield, even product type, may be varied. This can be done by imposing further selective environmental factors, or by genetic manipulation of the organism.

We regard salinity as a convenient, relatively easily manipulated and inexpensive selective factor, particularly appropriate for an Australian algal biotechnology industry.

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